THE IDENTIFICATION OF PETAL FLAVONOL GLYCOSIDES FROM CALTHA PALUSTRIS, LOTUS CORNICULATUS, AND TRAGOPOGON PRATENSIS

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Gary Owen Slaybaugh

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Approved by Committee:

Chairman

Earle L. Canfield
Dean of the School of Graduate Studies

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INTRODUCTION

Relationships based upon external, morphological characteristics have been the major botanical approach to plant classification for many years. Recently a number of other methods, including cytology, pollen morphology, and chemotaxonomy, have provided additional evidence of these relationships. Concerning chemotaxonomy, Erdtman (1963) stated that it was simple in principle. The chemotaxonomic method essentially consisted "of the investigation of the distribution of chemical compounds, or groups of biosynthetically related compounds, in series of related or supposedly related, plants." Techniques such as two dimensional paper chromatography, thin layer chromatography, and spectroscopy have made the chemotaxonomic approach a reliable indicator of plant relationships. Chemotaxonomy has also proven to be valuable, because the techniques involved have been independent of the older descriptive methods (Erdtman, 1963). But, Bate-Smith (1962) pointed out that the primary application of chemotaxonomy has been to aid in establishing the validity of the older descriptive methods of classification. He also predicted, "For a long time to come . . . chemical 'characters' must be related to existing systems . . . in the search for the ideal, natural system of classification."

Many chemical compounds have been investigated and

evaluated as to taxonomic importance. Bate-Smith (1958, 1962, and 1963) established that flavonoids were excellent compounds for such studies, because of their chemical stability, ease of precise identification, lack of use in cell activities other than cell wall formation, universal presence in plant tissues in sizable quantities, and great 'species specific' variance in detailed structure among one or more related or unrelated species. The diversity among flavonoids is due in part to the wide range of variation in the pattern of substituted compounds, such as oxygen-linked sugar molecules attached to the basic flavonoid structure called an aglycone. These compounds, which are termed flavonoid glycosides, are easily converted to aglycones and sugars by either acid hydrolysis or use of enzymes. The basic flavonoid structure is shown in diagram I of figure 3.

Bate-Smith (1962) observed that, although flavonoids were formed biogenetically by a common pathway of synthesis, little was known about the biosynthetic details or biochemical interrelationships of particular flavonoids. Nevertheless, he pointed out the taxonomic value of such data (Bate-Smith and Metcalfe, 1957; Bate-Smith, 1958, 1962, and 1963).

Because of the need for complete detailed flavonoid analysis and as an extension of the recent work

on distribution of flavonoid glycosides in petals, the purpose of this investigation was to study the kinds of pigments present in species of three families of Dicots and to establish evidence for an evolutionary flavonol sequence in the line leading to the Compositae. This was done by determining the chemical structure of the major flavonols present in the petals of these species. Selection of the specimens leading to the composite line was based upon the classification system proposed by Bessey, and others, as reported by Pool (1941) and Robbins, Weier, and Stocking (1964). Identification of the specimens was based upon the Engler and Pratl system of classification as reported in the eighth edition of Gray's Manual of Botany. The species used in this study were: Caltha palustris L., family Ranunculaceae; Lotus corniculatus L., family Leguminosae; and Taraxacum pratensis L., family Compositae.

REVIEW OF THE LITERATURE

Veclakantam, Seshadri, and Roa's investigation (1935) of the petals of Gossypium herbaceum exemplifies early chemotaxonomic work. They isolated and identified the flavonols of gossypetin, gossypetrin, and quercetin by standard chemical methods including extraction, crystallization, acetylation, filtration, hydrolysis, precipitation, and melting point determination.

Spectral analysis was also employed during this period and some aglycones could be identified by spectral curves. Aronoff (1940) reviewed the literature and attempted to correlate absorption spectra with aglycone structures. He stated that the basic curve of flavones and flavonols was due to the benzopyrone portion of the nucleus and that the B ring did not basically alter this curve. Diagram I of figure 3 shows the structure of flavone. Flavonol has the same structure except for a hydroxy group in the 3 position. He further maintained that the curve was caused by the presence of the oxygen on the phenone, and that the λ_{max} were altered by the hydroxy substitution pattern. Maxima (λ_{max}) are the wave lengths of greatest absorbance and appear as peaks on a spectral curve. An inflection is a pronounced plateau, which appears on the side of a peak. The maxima in the 300 to 400 mμ range are called Band I, and the maxima in the 200 mμ range are termed Band II. See figure 1 for an example of a spectral curve.

The research of Consden, Gordon, and Martin (1944) pointed out the value of paper chromatography as an analytic tool. They used descending chromatography to separate and identify microquantities of amino acids and indicated that the type of solvent used was a major factor for good separations. Their work served as a guide for other areas

of research. Partridge (1946) applied descending chromatography to the analysis of reducing sugars. Bate-Smith (1948) used descending chromatography and the solvent system of n-butanol: acetic acid: water (4:1:5) to separate anthocyanins, butein, and a flavone glycoside, and employed acid hydrolysis before he chromatographed the sugars. He introduced the method of direct application of a plant extract to chromatographic paper. In addition Bate-Smith (1949) tabulated R_F data for flavones and flavonols and concluded that flavonoid structure can be related to R_F values.

Wender and Gage (1949a) applied the descending chromatographic method to flavonoid microquantitative work reporting R_F values of nine flavones and flavonols and their color reactions to five chemical reagents.

Gage and Wender (1949b) reported the bathochromic shift behavior of seven flavones and flavonols upon the addition of a dilute solution of aluminum chloride, but it was Swain (1954) who first indicated that the shift was due to the chelating effect of aluminum with the orthohydroxycarbonyl groups. He stressed that there was a marked difference in behavior between 5-hydroxyflavones and flavonols in the presence of aluminum chloride. Jurd (1962) has summarized these shifts as follows: The 3 position hydroxyl group undergoes a +60 mμ shift of Band I

with or without a free 5-hydroxy group, and a 5-hydroxy-flavone or 5-hydroxyflavonol with a 3 position protected by sugar or a methoxy group undergoes a +20 to +45 mμ shift for Band I. A bathochromic shift refers to any shift in spectral maxima toward the longer wave lengths, and the term hypsochromic refers to a shift toward the shorter wave lengths upon the addition of reagents. Bathochromic values are denoted by a + and hypsochromic values are identified by a - sign. Figure 2 shows a bathochromic shift.

Mansfield, Swain, and Nordström (1953) discussed the value of dilute sodium ethoxide (NaOEt) solutions for the location of monohydroxy groups on the flavone nucleus during spectral analysis. In the presence of a 0.002 M NaOEt solution the monohydroxy groups in the 5, 7, and 4' positions underwent characteristic shifts of +50 to +60 mμ with corresponding increases or decreases of their λ_{max} for Band I. Nordström and Swain (1953) used this reagent during the identification of five flavone glycosides from Dahlia variabilis, and described a methylation technique for the location of sugar attachment points.

Jurd (1956) reported that flavonols and flavones with a free orthohydroxy system in the 3', 4' position underwo a characteristic shift of +16 to +29 mμ in the presence of ethanolic boric acid and fused sodium acetate

(NaOAc). Later, Jurd (1962) expanded the acceptable range to +15 to +30 mμ. Parks (1967) maintained that an o-dihydroxy group anywhere on the flavone nucleus could be detected by this method.

Jurd and Horowitz (1957) outlined methods by which NaOEt and NaOAc could be used to determine free 3, 7, and 4' hydroxy groups. Their work demonstrated that flavonols with hydroxyl groups at the 3, 4' positions were unstable in a 0.002 M NaOEt solution. If either of these groups were protected by a sugar or methoxy group, no degradation occurred. But compounds such as myricetin, which has a pyrogallol group (3', 4', 5' hydroxyls), and gossypetin with the hydroquinone system (5, 8 hydroxyls) were unstable whether or not the 3, 4' hydroxyl function was free. Band II underwent a +8 to +19 mμ shift in NaOAc, whenever the 7 position was hydroxylated, regardless of the presence or absence of other hydroxyls. If position 7 was protected by sugar or a methoxy group, no shift of this magnitude occurred. Jurd (1962) reviewed the spectral analysis data of flavonoids.

Harborne and Sherratt (1957) found that the artifact arabinose was produced when an anthocyanin glycoside was purified by a solvent containing hydrochloric acid. This degradation was avoided by using acetic acid.

Geissman (1962) stated that a flavonol was a

indicator of a more primitive condition and that flavones were indicative of a more advanced condition. He proposed (1962) that woody, primitive plants had the necessary enzymes to produce the more oxidized flavonoids such as flavonols. But, through mutations certain enzymes were irreversibly lost resulting in less woody plants with more advanced, less oxidized flavonoids like flavones. Only rarely were additional hydroxylation steps added by mutation.

Egger (1961) established the reliability of thin layer chromatography (TLC) for the identification of unhydrolyzed flavonol glycosides. He tested 14 glycosides of kaempferol, quercetin, and myricetin and concluded that the 3-monosides could easily be distinguished from 3-biosides and 3, 7-diglycosides by R_f values, the decisive R_f factor being the glycosidation pattern and not the aglycone hydroxy pattern. The aglycones of kaempferol, quercetin, and myricetin contain 3, 5, and 7 hydroxy groups. They differ from one another in that kaempferol has a hydroxyl group in the 4' position, quercetin (II) has an o-dihydroxy grouping in the 3', 4' positions, and myricetin (III) has hydroxyl groups in the 3', 4', and 5' positions.

Chandler and Harper (1961a) presented a method for absolute identification and location of sugars on most flavonoids by controlled hydrolysis using hydrazine,

permanganate, ozone, HCl, and acetic acid. A later work (1961b) employed molecular extinction coefficients for determining aglycone-sugar molar ratios.

Harborne (1965a) pointed out the value of 'species specific' flavonoid glycosides in describing a method by which oxygen-linked and carbon-linked glycosides could be distinguished by acid and enzyme hydrolysis. He found that the length of time, type of enzyme used, and resistance to acid hydrolysis characteristically revealed the position of glycoside attachment and the nature of the bond. Hedin et al. (1968) identified 10 new glycosides from the petals of Hibiscus esculentus using chromatographic, hydrolytic, and spectral methods. They described the occurrence of a 5-flavonol glycoside for the first time. They also reported a 5' glycoside.

Structural analysis has been carried out on flavonol glycosides from the petals of C. palustris and L. corniculatus, but no analysis has been reported for those of T. pratensis. Egger (1959) stated that the primary aglycone in C. palustris petals is quercetin with kaempferol present to a lesser extent. In a later publication Egger and Keil (1965a) demonstrated the presence of quercetin 3-galactoside and quercetin 3-galactoside-7-rhamnoside in the petals of the same species, but no kaempferol. They used thin layer chromatography and partial hydrolysis for

their identifications. The solvent system was water: ethanol: methyl ethyl ketone: acetyl acetone (13:3:3:1). Egger and Keil (1965b) did an extensive flavonol survey of the subdivisions in the family Ranunculaceae, including C. palustris, using the previously reported TLC method (Egger, 1961). They reported that kaempferol glycosides were associated with white flowers and quercetin glycosides with carotenoid-colored flowers. The kaempferol-quercetin pattern of distribution in white and yellow flowers indicated to them that a study of aglycones among the Ranunculaceae would be insignificant for chemotaxonomic purposes. Therefore, they conducted a glycoside survey of 50 species of Ranunculus and concluded that all genera exhibited 3 position glycosides and 3, 7-position diglycosides with 'species specific' glycosidic patterns. However, other types of substitutions have been reported among the Ranunculaceae such as 3 position glucuronic acid and 8 position glycosides (Egger and Keil, 1965b); isorhamnetin (quercetin 3'-methyl ether) (Harborne, 1957); and a flavonol complex, quercetin 3-caffeoylsophoroside-7-glucoside (Harborne, 1965a).

The hydrolysis method was used by Harborne (1965b) for identifying the structure of the quercetagetin 3-glucosides from the petals of L. corniculatus. The resulting aglycones were quercetagetin and quercetagetin

7-methyl ether (IV). Quercetagetin has a hydroxy group in the 7 position. Among the other Leguminosae Karrer (1958) and Harborne (1965a and 1965b) recorded the occurrence of the aglycones kaempferol, isorhamnetin, pratoletin (3, 5, 8, 4'-tetrahydroxyflavone), quercetin, and quercetagetin. Leguminosae glycosides typically exhibited the 3 glycoside or 3, 7-diglycoside pattern. Harborne (1965a) concluded that the wide occurrence of the 3, 7-diglycoside showed that there was no broad taxonomic significance for this pattern of substitution and predicted that it should be present in unrelated families. He also said that glycosidic studies of this pattern of substitution at the generic and species level should be significant. This was verified by Egger and Keil (1965b). Thus it is apparent that substitution patterns other than the 3, 7-disubstitution grouping are chemotaxonomically important within a group.

Among the Compositae Karrer (1958) reported the presence of the aglycones quercetin, quercetagetin, and patuletin (quercetagetin 6-methyl ether). The sugars were located in the 3 and 7 positions.

Concerning the evidence for an evolutionary flavonoid sequence, Lawrence et al. (1939) established a trend for three anthocyanins. Cyanidin was considered to be the primitive configuration, which gave rise to the

more advanced pelargonidin, delphinidin, or methylated forms by one or more additional biogenetic steps. However, Bate-Smith (1962) considered that the loss of such steps denoted advancement. Harborne (1963) resolved this conflict by pointing out that the added biogenetic steps were present as a result of natural selection, which counteracted mutations. This was because the blue flower colors were produced by biosynthetically more complex anthocyanins which were more attractive to pollinating insects than primitive cyanidin types. He noted that cyanidin-3-glycosides rarely produced blue colors, but that the more abundant co-pigmented, acylated, methylated, anthocyanin triglycosides did. Kaempferol was often the co-pigment. Sometimes quercetin was also identified as the co-pigment for nitrogenous anthocyanins (Blank, 1947).

Evidence for an evolutionary flavonol sequence for anthocyanin-colored petals was established by Klein and Hagen (1961). They discovered that the white and anthocyanidin-colored sepals of Impatiens balsamina contained 3', 4' o-dihydroxy cyanidin and quercetin, whereas the more highly evolved petals contained kaempferol and pelargonidin (4'-OH), and malvidin and myricetin (3', 4', 5' O-substituted flavonoids). When detached petals were grown in a liquid culture medium, they produced cyanidin and quercetin as well. The

concluded that this phenomenon could be ascribed to the partial inhibition of biosynthetic steps, which normally converted a precursor to cyanidin and quercetin. They reasoned that it was easier to interfere with a highly specialized process than a more primitive one. Accordingly the appearance of cyanidin and quercetin in cultured petals was due to the disturbing of the more specialized steps, which dealt with the production of 4'-mono- and 3', 4', 5'-trihydroxy derivitives. Since both anthocyanins and flavonols were similarly affected they hypothesized that the hydroxylation pattern was modified in a compound which served as a precursor to both, and the appearance of cyanidin and quercetin was interpreted as evidence of a return to a more primitive 3', 4' hydroxy pattern. Impatiens is a Dicot but is not in the composite line. Clevenger (1964) stated that the simultaneous production of malvidin and myricetin in Impatiens was controlled by one dominant gene. Blank (1947) indicated that the transformation of chromogen to anthocyanin was linked to a definite gene in petunias. If the gene was missing, the flavone-like compounds were not changed into anthocyanins. Petunias are also Dicots and are in the same line of evolution as Impatiens. Birch (1963) proposed that the [illegible] direct route to anthocyanin formation was through a flavonol precursor. Parks (1965) studied mutant strains of

cotton plants (Gossypium) and noted that the blocking of gossypetin and quercetagetin pathways early in biosynthesis of one strain caused the shunting of precursors which were then used to form kaempferol. In a second mutant strain there was decreased gossypetin production and a corresponding increase in quercetin and kaempferol production. Gossypium is in the same order as Impatiens.

MATERIALS AND METHODS

C. palustris petals were collected in April near the Indiana Dunes State Park, and North Barrington and Wauconda, Ill. in swampy areas. L. corniculatus flowers were collected in the latter part of May along U.S. 41, south of the west turn off for Ind. State Route 2. T. pratensis flower heads were collected in early June near Naperville, Ill. All flowers were dried at 40° to 50° C, and the drying time varied from two to five days. The petals were stored in envelopes until used.

Petal pigments were extracted with glacial HCl diluted to 1% with absolute methanol (volume/volume). The crude extracts were placed in silica cuvettes and absorption spectra were obtained in a Beckman model DU Spectrophotometer with synchronized log span recorder. The spectrophotometer measures the amount of light absorbed by the pigment at each wave length as the instrument scans all wave lengths from 670 to 240 mμ and an absorption

spectrum is recorded by synchronized strip graph paper and ink pen. The shape of this curve and the wave length at which maximum absorption occurs are very precise ways of identifying the compound. By using various reagents the point of maximum absorption may be shifted and these changes give definite information about the structure of the compound under study.

Each crude extract was streaked with a pipette on 23 by 57 cm No. 3 MM Whatman chromatography paper, and developed by mass descending chromatography overnight using solvent a (table 1). As the solvent moves down the paper the more soluble pigments in the mixture are carried near the solvent front and the less soluble lag behind. After drying, the visible pigment bands were cut from the filter paper, eluted from the paper with absolute ethanol, and absorption spectra were again taken to help determine the nature of the purified pigment.

Each eluate was streaked on 22 by 22 cm No. 3 MM Whatman paper and developed ascendingly using various solvent systems. Purification of L. corniculatus flavonols was accomplished by employing solvents b, c, and d. The L. pratensis flavonol was purified by using c, and the C. palustris separation was achieved by using b. Whenever large quantities of eluate resulted, the volume was reduced by evaporating in vacuo at less than 40° C.

Table 1. Chromatographic solvent systems

	Solvent mixture	Volume ratio
a	n-Butanol:gl acetic acid:water	4:1:5
b	Water saturated with phenol	———
c	Gl acetic acid:water	15:85
d	Isopropanol:water	6:4
e	Cyclohexane:ethyl acetate	1:1
f	Chloroform:methanol	7:3
g	Water	———
h	n-Butanol:ethanol:water	4:1:2.2
i	n-Butanol:benzene:pyridine:water	5:1:3:3
j	Phenol saturated with water	———

Each flavonol glycoside was double checked for purity by employing thin layer chromatography (TLC). The TLC matrix is a plaster of paris-like substance, which is usually placed on a glass plate. The plates were prepared according to the method of Lees and DeLuria (1962). Mallinckrodts' SilicAR TLC-7GF was mixed with water at a 1:2 ratio (gm/ml). The plates were activated by heating at 100° C for one hour before use. Each eluate was then spotted with a micropipette, and developed using solvents e and f (Grant and Zalite, 1966). The plate was dried

between runs. After purification, spectral curves were determined in absolute ethanol; for ethanolic solutions containing aluminum chloride (Swain and Nordström, 1956); sodium ethylate (Mansfield et al., 1953); saturated boric acid: sodium acetate (Jurd, 1956); and sodium acetate (Jurd and Horowitz, 1957).

Each eluate was then hydrolyzed by heating for two hours in the dark using 2.0 N HCl in methanol (1:1) (Hedin et al., 1968; and Furuya and Galston, 1965), and the hydrolysate was streaked on sheets of Whatman No. 3 MM paper. The ascending chromatogram was developed with water. Using water as a solvent, the sugar moved close to the solvent front, while the aglycone moved very little. After drying, the region of the paper containing the aglycone was cut off, eluted from the paper with absolute ethanol, and spectral curves determined in the manner previously described for the flavonol glycosides.

A nine cm wide, 22 cm length of each chromatogram containing sugar hydrolysate was cut out and stored for analysis later. At that time one of the eight cm margins on each strip was cut to a point. The other end was placed in water, the chromatogram developed, and the eluate collected at the pointed end. Three of the six eluates and 15 known mono-, di-, and trisaccharides were then spotted with a micropipette on 46 by 57 cm No. 1 Whatman paper.

Four chromatograms were spotted and each sheet was then developed employing solvents a, h, i, and j. Sugar identification was achieved by comparing R_G values with hydrolysate R_G values after spraying with aniline-H-phthalate and heating for 10 min. at 110° C. R_G values are calculated by dividing the distance that glucose moves into the distance traveled by the other sugars. This method was used, because the solvent front was usually indistinguishable.

It later became necessary to hydrolyze commercially prepared D(+)raffinose in the manner previously described. The resulting hydrolysate was spotted on No. 1 Whatman paper with the known sugars of L(+)arabinose, D-arabinose, D-glucose, D(+)raffinose, L-sorbose, sucrose, and D-xylose. The chromatogram was developed ascendingly using solvent j.

RESULTS

The spectral maxima obtained during this investigation are summarized in tables 2, 3, and 4. The spectral shifts in conjunction with sugar identification will be used to determine detailed flavonol structure. Alternative structures will be discussed where appropriate. Only the yellow flavonoids that were visible during mass descending chromatography were isolated and studied spectrally. The numbering system for flavonoids is shown in I of figure 3.

Table 2. Spectral maxima of the flavonol glycosides and aglycones of C. palustris

Compound	Maxima absolute ethanol	$AlCl_3$	Sodium ethoxide (NaOEt)	Fused sodium acetate (NaOAc)	Boric acid & NaOAc
II[a]	364	408	416	379	386
		360[b]	332		
	296	302[b]			
	257	259	274	268	263
	373	437	415 (u)[c]	386	394
		366[b]	330		
		302[b]			
	255	269	295	258	261
III	377	431	406[b] (u)	398	392
		366[b]	327	337	
		305[b]		277[b]	
	255	267	298	254[b]	259
	375	444	408 (u)	387	394
			332	275[b]	
	255	270	298	257	261

a The spectral data for each glycoside is summarized after the Roman numeral followed by aglycone data underneath.

b Denotes an inflection.

c Refers to the chemical breakdown of the flavonol in the presence of this chemical.

Table 3. Spectral maxima of the flavonols of *L. corniculatus*

Compound	Maxima absolute ethanol	$AlCl_3$	(NaOEt)	(NaOAc)	Boric acid & NaOAc
IV	380	446	422 (u)	403	402
		371	339	339	359
	274[b]	310[b]	296	279	282[b]
	260	272		257[b]	265
	379	444	425 (u)	394	401
		369	338	332	
	272[b]		294	276	
	259	271		260[b]	264
V	382	467	376	373	378
	348[b]	381			
	274	285	287		
	262[b]				257
	384	455	374	374	379
	343	379			
	278[b]				
	260	276	284		251
VI	352	420[b]	367		372
		367		326[b]	
	274	305[b]			
	262[b]	282	284	282	274
	361	403	406	371	385
		367[b]	332[b]		
	298[b]	302[b]			
	257	270	272	264	263

Table 4. Spectral maxima of the flavonoids of *T. pratensis*[a]

Compound	Maxima absolute ethanol	$AlCl_3$	NaOEt	NaOAc	H_3BO_3 & NaOAc
VII	348	390	384	354	382
		355			
	268	301[b]			
	257[b]	274	272	269	265
VIII	356	424	405	381	386
		354			
	268[b]	302[b]	331		
	257	272	278[b]	271	263
IX	341	389[b]	342	364	370
	292[b]	351			
	268[b]	300	268[b]		
	255	276	256	268	262

[a] There are no aglycone spectral data for VI and VIII as well as no glycoside data for VII.

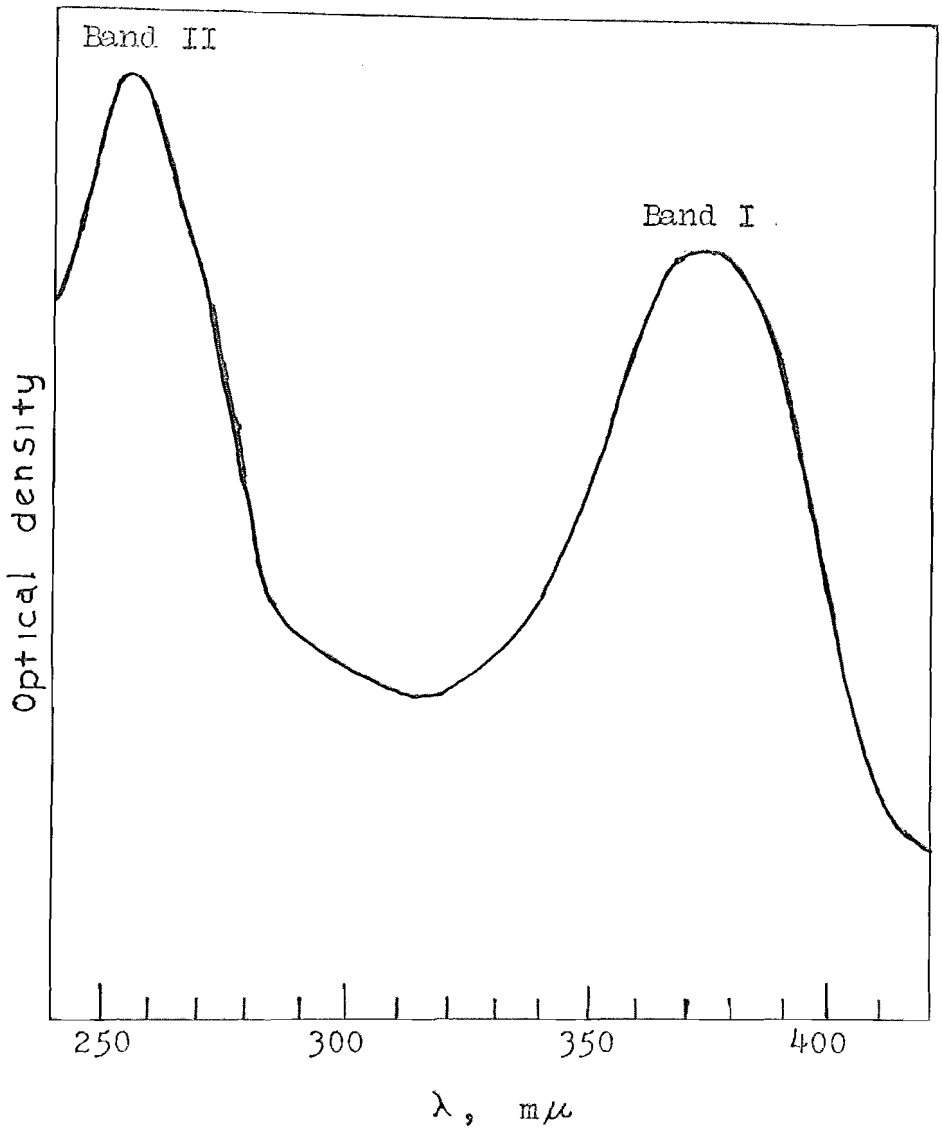


Figure 1. Ethanolic spectrum of the aglycone for quercetin 3-raffinoside (II).

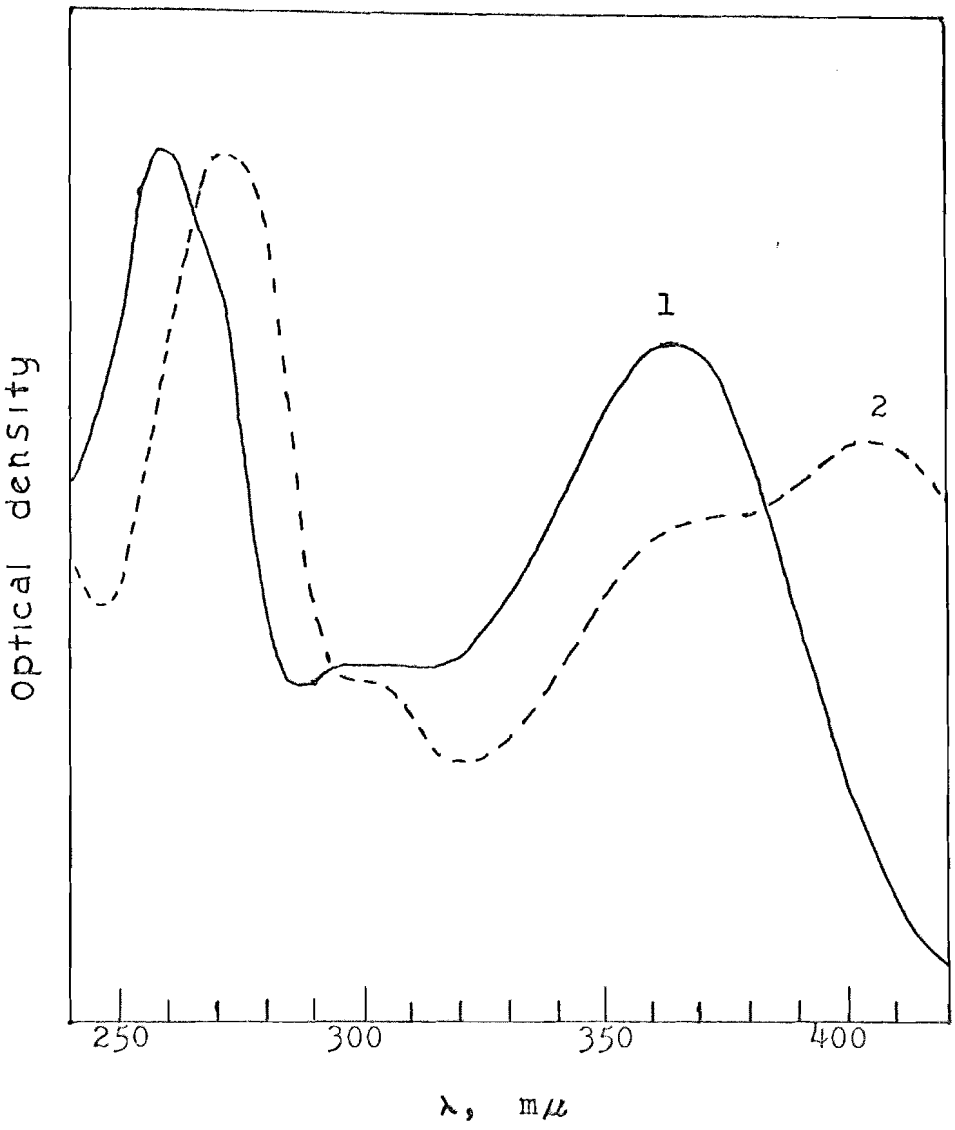
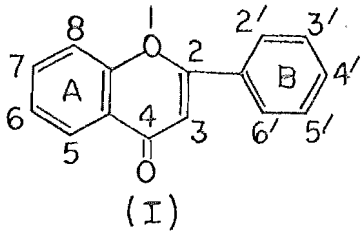
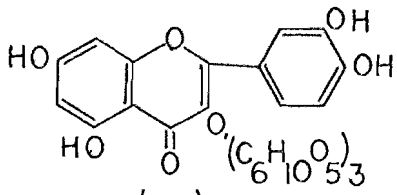


Figure 2. Spectra of 5, 7, 2', 3', 4'-pentahydroxy-3-methoxy-5,4'-diglycoside aglycone (VI) in (1) ethanol ———— , (2) aluminum chloride ---------.



(I)



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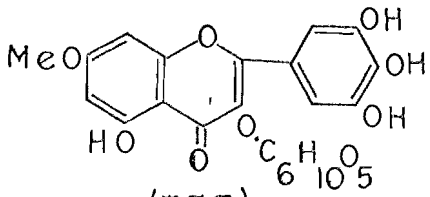

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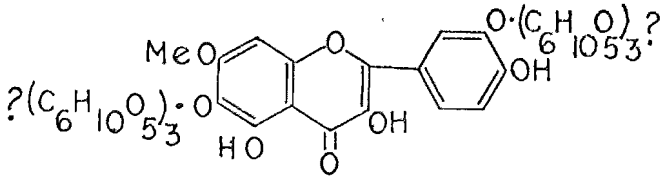
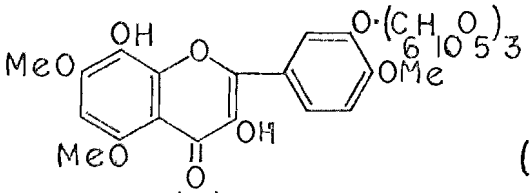
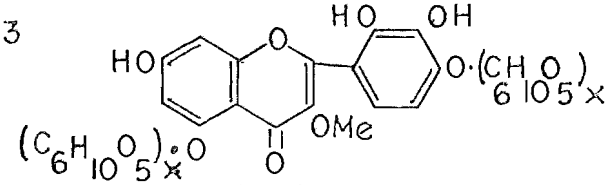


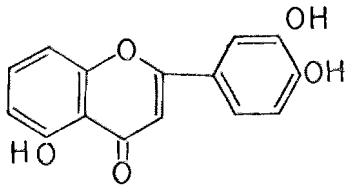
(IV)



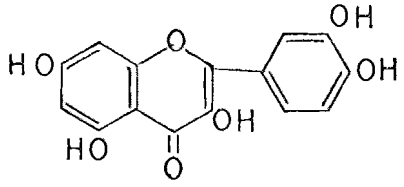
(V)



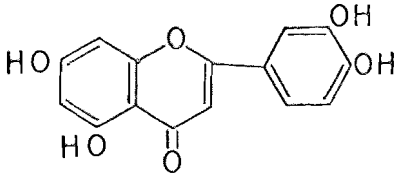
(VI)



(VII)



(VIII)



(IX)

Figure 3. Major petal flavonoids of C. palustris, L. corniculatus, and T. pratensis.

C. palustris flavonols.

Quercetin 3-raffinoside (II). The +44 and +64 mμ Band I shifts in $AlCl_3$ indicated that there was a hydroxy group at the 5 position and a 3 position masked by sugar. The ethanolic reading 364 λ_{max} was consistent with the reported hypsochromic shift for a protected 3 position (Jurd, 1962). The band I shift in NaOAc revealed that there was a hydroxy group at either the 3 or 4' position. The Band II shift in NaOAc showed that there was a hydroxyl group at position 7. The stable glycoside and unstable aglycone established that either the 3 or 4' position was protected by sugar. The shift of Band I in NaOEt without decreased intensity indicated that the 4' position was free. Thus, the sugar was attached to position 3. The boric acid/NaOAc shifts showed that an o-dihydroxyl group was present. The choice was 3', 4' or 4', 5', because the ethanolic maxima did not support the choice of the 6, 7 or 7, 8 position. Sugar analysis revealed the presence of sucrose and xylose in the hydrolysate, thus, two sugar attachment points may have existed. The 3, 5' or 3, 3'-di-glycoside of myricetin was considered possible, because the ethanolic aglycone maxima and the stability of such a glycoside in NaOEt supported this choice. See figure 1. But the hydrolysate of raffinose yielded sugar fragments of sucrose and xylose, indicating that raffinose only may be

attached at position 3 or the other positions as well. The quercetin aglycone was chosen, because the literature supports this decision (Egger and Keil, 1965a). They reported quercetin 3-galactoside in C. palustris petals, but no myricetin compounds. Jurd and Horowitz (1957) recorded the λ_{max} of 370, 257 for quercetin in ethanol whereas Clevenger (1958) reported the λ_{max} of 374, 310^{b}, 259 and λ_{max} 376, 256 for ethanolic quercetin. These maxima corroborate the choice of quercetin. Additional chromatographic or acetylation and methylation studies are needed to confirm the choice between the myricetin or quercetin glycoside.

7-methoxymyricetin-3-glycoside (III). Ethanolic maxima supported the myricetin assignment (λ_{max} 376, 255), since there was no quercetin-type inflection in the 260 μ range (Jurd, 1962). But rhamnazin (?, 3'-di-O-methylquercetin) must be considered. Its λ_{max} are 375, and 255 in ethanol. The band I shifts in NaOAc indicated that there was either a free 3 or 4' hydroxyl group. No 7 position hydroxy group was present, due to the lack of band II shifts in NaOAc, thus, it was methylated (Mabry et al., 1963). The instability of the glycoside and aglycone in the presence of NaOEt established that either the 3 or 4' hydroxyl groups were free, or the flavonol structure was myricetin with hydroxy groups at the 3', 4', 5'

positions, or gossypetin with unprotected 5, 8 hydroxy groups. The ethanolic maxima support the choice of myricetin or quercetin but not gossypetin. The spectral shifts in boric acid/NaOAc demonstrated an o-dihydroxy function at either the 3', 4' or 4', 5' positions, thus eliminating rhamnazin as a possibility, since its 3' position is methylated. The +54 and +69 mμ $AlCl_3$ shifts suggested a free 5-OH and a 3 position masked by sugar. Some variance from the +45 mμ mixed 3, 5-OH function and free 3-OH (+60 mμ) occurs in the literature. For example, rutin (quercetin 3-rutinoside) exhibits a +53 mμ for a mixed hydroxy function (Gage and Wender, 1949). And patuletin (6-methoxyquercetagetin) exhibits a +70 mμ for a 3 hydroxy group (Harborne, 1965b). Since there was a protected 3 position, the instability of the glycoside in NaOEt was caused by the hydroxy groups in the 3', 4', 5' positions, verifying the myricetin assignment. This compound has not been reported from C. palustris petals. This compound may be similar to the myricetin 3-digalactoside described by Hattori (1962) in Betula, since either galactose or glucose was the sugar present in the hydrolysate.

L. corniculatus flavonols.

3-methyl ether of quercetagetin-6 or 3'-raffinoside (IV). The spectral data for this flavonol agreed with the

values reported by Harborne (1965b). The $AlCl_3$ Band I shifts revealed a free hydroxy group at the 3 position. The glycoside instability in NaOEt demonstrated hydroxy groups at the 3, 4' positions. Ethanolic maxima supported the quercetagetin assignment, but not the choice of gossypetin or myricetin. The lack of a Band II shift in NaOAc verified the presence of a methoxy group at the 7 position. The +22 mμ Band I shift in $NaOAc/H_3BO_3$ disclosed that there were hydroxy groups at either the 5, 6 or 3', 4' positions. Thus, the sugar attachment positions were in either the 5, 6 or 3' position. These attachment points have not been reported in the literature. Flavonols with glycosidation in the 5 position are rare (Hodin et al., 1968). The hydrolysate contained sucrose and xylose, which supports the choice of a raffinose compound. Harborne's quercetagetin 7-methyl ether 3-galactoside assignment did not then agree with my data. Further study would help confirm the location of the sugar on the flavonol.

5, 7, 4'-trimethoxygossypetin-3'-raffinoside (V).

The ethanolic readings were closest to the λ_{max} 380, 350, 278, 262 for gossypetin 7-glucoside. The aglycone λ_{max} agreed closely with the gossypetin values of 376, 341b, 278, 262 (Jurd, 1962). This data eliminated quercetin, myricetin, quercetagetin, and 7-methoxyquerce-

tagetin as possible aglycones. The $AlCl_3$ shifts revealed the presence of a hydroxy group in the 3 position. The reason for the +85 mμ shift was not known, since the 3-OH for gossypetin and most other flavonols is around +60 mμ. Although the $AlCl_3$ curves were not typical for either a free 5 hydroxyl; a mixed 3, 5-OH function; or a free 3, 5 hydroxy grouping, it was deduced that both were free, due to the lack of the necessary hypsochromic shifts of Bands I and II. Such hypsochromic shifts indicate a protected 3 or 5 position hydroxyl grouping in ethanol (Jurd, 1962). The $AlCl_3$ curves were atypical, because, although they closely resembled a free 5-OH or a mixed 3, 5 hydroxy function curve, they differed from such curves in that the Band IIa was missing (Jurd, 1962). The disappearance of Band II in the presence of NaOAc, instead of the usual shift or no shift of a maxima, disclosed that there was a methoxy group at position 7. This interpretation of a spectral curve has not been reported in the literature. This conclusion was confirmed, when the lack of bathochromic shifts in the presence of boric acid/NaOAc indicated that there was no o-dihydroxy function at the 7, 8 or 3', 4' positions. Thus, the 7 position was methylated as well as one of the 3', 4' positions. The 8 position was deduced to be free, because the ethanolic maxima of gossypetin 8-glucoside did not agree with the pigment under study,

whereas gossypetin with a free 8 hydroxyl did (Jurd, 1962). Interpretation of the hypsochromic shifts in boric acid/NaOAc, NaOAc, and NaOEt has not been reported. Previous work has established that a lack of a bathochromic shift of Band I in NaOAc indicates that either the 3 or 4' position or both may be protected (Jurd, 1962). Also, a protected 3 or 4' position exists for a flavonol, if it is stable in NaOEt. Both conditions existed in the experimental pigment. Since the $AlCl_3$ shift proved the presence of a free 3 hydroxyl, the 4' position must be methylated. A contradiction of the data exists. It was deduced that the 5 and 8 hydroxyl groups were free. Such a condition is unstable in NaOEt, but the pigment under study was stable. Thus, either the 5 or 8 position was protected. The choice was the 5 position, due to the atypical $AlCl_3$ curves. But the ethanolic hypsochromic shifts of Bands I and II did not correlate with the expected -5 to -15 mμ shifts of both Bands for a 5-methoxy group (Jurd, 1962). Thus, the 5-methoxy assignment must be considered tentative. The hydrolysate contained sucrose and xylose, indicating that raffinose was attached at the 3' position. A protected 3 position was already eliminated. The data raised two questions that warrant additional research. Why causes the abnormally large +95 mμ shift in $AlCl_3$? And what caused the hypsochromic shifts?

5, 7, 2', 3', 4'-pentahydroxy-3-methoxy-5, 4'-diglycoside (VI). The +68 mμ inflection in $AlCl_3$ suggested that the 3 position was hydroxylated. The +42 mμ aglycone shift (see figure 2) indicated that there was either a freed 5-OH or a freed 5-OH with a methylated 3 position. The latter was correct, since the former was based upon an inflection only, and the +68 mμ shift would have persisted in the aglycone spectrum as a maximum, if the 3 position was hydroxylated. The lack of a definite shift in $AlCl_3$ in the glycoside and the presence of one in the aglycone revealed that the 5-OH was glycosidated. The Band I ethanolic glycoside and aglycone maxima also supported this conclusion. The ethanolic aglycone Band I maximum was identical to the hypsochromic shift for 3-O-methyl quercetin (Jurd, 1962). Band II of quercetin in ethanol is not appreciably affected by a 3 methoxy substitution. The reason for my atypical Band II ethanolic shift was not known, (262 to 257 λ_{max}) but the 3 methylated condition was still considered to be the best choice. No shift for Band I in NaOAc established that the 3 or 4' was protected. The Band I shift for the aglycone in NaOAc demonstrated that there was a free hydroxy group at 4', since the 3 position was methylated. Thus, the 4' position was glycosidated. The aglycone Band I shift in NaOEt with no intensity decrease confirmed the presence of a glycosidated

4' position. The Band I shift of the aglycone in ethanol disclosed that a protected hydroxy group was free, probably at the 4' position. This shift has been reported by Jurd (1962). The stability in NaOEt verified the 3 methoxy group. The Band II shifts in NaOAc showed that there was a hydroxy group in the 7 position. The Band I shifts in boric acid/NaOAc established the presence of an o-dihydroxy system in the glycoside. The 7, 8 position was eliminated, due to the instability of the 5, 8-OH function in NaOEt. Since the 4' position was glycosidated the pigment cannot be myricetin, since no o-dihydroxy function would occur with a protected 4' position. The stability of the aglycone and instability of myricetin in NaOEt also supported this conclusion. Thus, the o-dihydroxy function must be in the 2', 3' position. This has not been reported before. Methylation of the 5 hydroxy position causes hypsochromic shifts of Bands I and II in ethanol. I do not know whether glycosidation of the 5 position would cause the same kind of shift. On the basis of the glycoside data such a shift could exist for Band I, but Band II failed to shift as expected for a methylated condition. A flavonol having sugar at the 5 position has been reported (Nedin et al., 1968). The hydrolysate contained sucrose and either xylose or arabinose, but their exact placement on the aglycone was not determined. This compound may be

impure, since two pigments with R_Fs of 0.58 and 0.59 in acetic acid: water (15:85) were detected. Both had identical ethanolic glycoside curves, each reacted differently in the presence of ammonia fumes and UV light. The 0.59 R_F compound only was chosen for further investigation. An unidentified anthocyanin was observed to be heavily concentrated at the front edge of the "keel" formed by the lower petals of Lotus.

E. pretensis.

Although VII and VIII were obtained from separate chromatographic runs, they had identical ethanolic glycoside maxima and were thus considered to be identical. Analysis of their spectral curves revealed, however, that they are different pigments.

VII. The Band I shift in $AlCl_3$ indicated that there was a free 5 hydroxy group. The decreased intensity of Band I in NaOEt suggested that the 4' position was not free. The boric acid/NaOAc shift verified the presence of an o-dihydroxy function at the 3', 4' positions. The +6 mμ Band II shift in NaOAc was not considered proof of a 7 position hydroxy group. The ethanolic λ_{max} of 262 and 257^{sh} confirmed that there was a 3', 4' o-dihydroxy system. No conclusions could be reached as to whether it was a flavonol, but aglycone data would help resolve this problem.

Quercetin (VIII). The $AlCl_3$ shifts disclosed that there was a 3 position hydroxyl group. Thus, the compound was a flavonol. The Band I shift without decreased intensity in NaOEt established that the 4' hydroxyl was free. Since the compound was stable in NaOEt, either the 3 or 4' position was protected, which is clearly contradictory to the previous conclusions. The reason for this contradiction was unknown. The NaOAc Band II shift revealed a hydroxy group in the 7 position. The boric acid/NaOAc shift indicated a free 3', 4' o-dihydroxyl system. This was consistent with the assignment of quercetin as the aglycone. The hydrolysate contained sucrose and either arabinose or xylose. No sugar attachment points are known.

IX. No flavones or flavonols matched the ethanolic values of the glycoside (Jurd, 1962). No aglycone spectral readings were taken, due to loss of the aglycone during chromatographic purification. Subsequent mass descending separations and spectral work never disclosed the presence of this compound again. Apparently a degradation problem was involved, because a mass descending separation nine months later revealed that there was no visible yellow pigment of any kind in the original T. protensis petals. The Band I shift in $AlCl_3$ indicated that there was a 5 hydroxy group. The Band I shift in NaOAc established

that there was either a free 3 or 4' hydroxy function. The Band II shift in NaOAc demonstrated that there was a free hydroxyl group in the 7 position. The stability of the glycoside in the presence of NaOEt signified that either the 3 or 4' position was protected or that there was no hydroxyl group at position 3. The Band I shift in H_3BO_3/NaOAc verified the presence of an o-dihydroxy function, probably at the 3', 4' positions. The λ_{max} of 268^b, 255 supported this conclusion. The low ethanolic λ_{max} of 341 cannot be easily explained, if it is assumed that the aglycone was quercetin, but such a possibility exists. Probably the aglycone was a flavone, but additional research is needed to confirm the choice of aglycone.

DISCUSSION

No 4'-monohydroxysubstitution pattern was observed in any of the flower petals, but all contained the 3', 4' o-dihydroxy pattern. This has been interpreted by a number of investigators to be a primitive condition for the [illegible]. The presence of quercetin in C. palustris and advanced T. pratensis indicated that there was no evolutionary selection away from this compound as was the case for its corresponding anthocyanin, cyanidin. The modification of quercetin in Lotus petals to form 3', 4' o-dihydroxy quercetagetin, gossypetin, and a 2', 3', 4'-trihydroxy substituent all of which are methylated may be

evolutionarily significant. The presence of an anthocyanin at the tip of the "keel" which conceals L. corniculatus reproductive parts probably serves as a pollinator guide for insects. One or more of the flavonols could have acted as a co-pigment for the anthocyanin, therefore providing a basis for evolutionary selection of flavonols built from quercetin. This would also explain why no detectable amount of quercetin was observed, since it was readily converted into other flavonol end products or an anthocyanin as proposed by Birch (1963). An equally valid interpretation for the greater variety of flavonoids in L. corniculatus than in C. palustris or T. pratensis is to assume that Lotus (and the order Rosales) occupies a more central position among the Dicots than either of the other two (Bate-Smith, 1962). This conclusion is supported by a systematic study of certain primitive and advanced morphological characters made by Sporne (1954).

Although myricetin has not been reported from the petals of Compositae, chance mutation must be responsible for its presence in the composite line, since myricetin is sporadically distributed among the specimens of this line. The randomness of the observed 6 and 2' hydroxy substitutions among the species of the composite line reveal that these positions are not significant from a broad taxonomic viewpoint. The constant association of

the 3', 4' o-dihydroxy pattern with carotenoid-colored petals, but not with white petals may be evolutionarily significant. White and anthocyanin-colored Impatiens sepals produce quercetin, but their white or anthocyanin-colored petals do not. This indicates that the advanced petal biogenetic mechanism shunts the o-dihydroxy 3', 4' precursor into alternate anthocyanin pathways and other forms of flavonols. This shunting process did not operate in the yellow-colored petals of this investigation, but did in the related white-petaled Ranunculaceae (Egger and Keil, 1965b) where kaempferol was formed instead of quercetin. Therefore, the presence of o-dihydroxy groups in the B ring indicates that the carotenoid association allows the expression of this substitution pattern in flower petals instead of the observed inhibition in white and anthocyanin-colored petals. Possibly the o-dihydroxy grouping is an important configuration for the yellow coloring in flower petals, such as found in quercetagetin and gossypetin, or for co-pigmentation. Therefore, selection for this substitution pattern may explain its presence in all of the flavonols under study. Gossypetin has been found only in the intermediate groups of Gossypium and Hibiscus of the order Malvales. The only composite line species to contain a gossypetin derivitive was reported for the first time in this thesis. This

sporadic occurrence in the unrelated legume and mallow groups may be due to chance mutation and is presently not considered enough evidence for reclassification. Note that if one assumes that the order Rosales which contains Lotus occupies the central position described by Bate-Smith (1962), it allows a much closer degree of relationship between the orders Rosales and Malvales than one which shows both groups descending from the order Ranales (Pool, 1941) that contains no reported gossypetin compounds. Since complex anthocyanins are selected for by their attractiveness to insect pollinators, it could be assumed that the complex gossypetin compound acts in the same manner or that its selection is due to co-pigmentation with the anthocyanin. Neither assumption has been proven. No evolutionary trend could be inferred from the unusual sugar attachment points or sugar moiety for Lotus pigments. Instead they represent a 'species specific' phenomenon.

SUMMARY

The purpose of this investigation was to establish evidence for an evolutionary flavonol sequence in the line leading to the Compositae by identification of the flavonol [illegible] of the Dicots Caltha palustris, Lotus corniculatus, and Arctostaphylos pungens. This purpose was achieved [illegible] that five new [illegible]

tentatively identified and the presence of a flavonol aglycone in T. pratensis was established. This knowledge adds significantly to the existing flavonol data, but additional evidence is needed to verify the evolutionary sequence among these specimens.

In this investigation petal extracts were purified by paper chromatography, visible pigments eluted from chromatograms, and eluates studied spectrally to determine the detailed structure of the major flavonols. The hydrolyzed sugars were isolated and identified.

The five glycosides were quercetin 3-raffinoside and myricetin 7-methyl ether-3-galactoside or glucoside from C. palustris; and 5, 7, 4'-trimethoxyprocyanidin-3'-raffinoside, 3-methoxy-5, 7, 2', 3', 4'-pentahydroxyflavone-5, 7'-diglycoside, and 7-methoxyquercetagetin-6 or 3'-raffinoside from Lotus corniculatus. The flavonol aglycone quercetin was found in T. pratensis.

This investigation suggests that the following research could be done. Complete identification of the T. pratensis flavonoids is needed. Only then could an evolutionary trend from the primitive flavonol to advanced flavone be confirmed or rejected. Rf values, and acetylation and methylation studies of these pigments would provide additional evidence for their structure. [illegible]

the unusual hypsochromic shifts encountered during the investigation of the gossypetin derivitive.

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